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PRINCIPAL INVESTIGATOR: George W. Sledge, M.D.

CONTRACTING ORGANIZATION: Indiana University
Indianapolis, Indiana 46202-5167

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Introduction

The goal of this research project was to develop a novel targeted drug delivery system which would allow delivery of drugs directly to cancerous breast tissue without delivering significant amounts of drug to other parts of the body. The systems described in this research proposal utilize biodegradable nanoparticles based on poly(lactic-co-glycolic) acid (PLAGA) containing paclitaxel (Taxol) or doxorubicin (adriamycin). The nanoparticles were prepared according to a novel technique which incorporates poly(ethylene glycol) (PEG) into the nanoparticles. Addition of PEG alone reduces the uptake of the nanoparticles by the reticuloendothelial system and increases their circulation time. In addition, the PEG arms of the nanoparticles were to be conjugated to monoclonal antibody (MAb) to HER-2/neu, which would selectively bind to breast cancer cells expressing the HER-2 extracellular domain, thereby allowing the nanoparticles to be delivered and targeted directly to cancerous breast tissue. Some nanoparticles are also prepared using polymers that are graft copolymers of PEG and PLA/PGA. All formulations of nanoparticles were to be prepared in order to be of a size less than 750 nm in diameter, preferably less than 300 nm.

Statement of Work

The key research activities of the first 12 months of this project included the development of nanoparticle formulations containing taxol and adriamycin. Although verification was received by the Principal Investigator some time ago from Bristol-Myers Squibb and Pharmacia & Upjohn that they would be able to provide taxol and adriamycin, respectively, for this project it has been quite difficult to actually receive these materials. We have received and purchased taxol but the adriamycin has not been received as this report is being written. Therefore nanoparticles have been prepared and evaluated containing taxol but no formulations have yet been prepared containing adriamycin. There were also repeated delays in obtaining the HER-2 antibody from Genentech, so the antibody was finally obtained approximately one month ago. The delay has not been a disadvantage, however, because during that time we have had the opportunity to fine-tune the nanoparticle preparation techniques far beyond that which was originally described in this proposal. The formulation work with taxol is progressing very well and, because of our experience with a variety of formulation techniques, we feel that the nanoparticles containing adriamycin will be able to be prepared and optimized very quickly, once the drug is received.

Preparation Techniques for Biodegradable Nanoparticles

The preparation of submicron PLAGA particles containing an active agent poses serious challenges that are not necessarily present when preparing larger diameter microparticles. The optimum formulation would satisfy the following:

- Submicron size production with a high yield (>90%)
- High encapsulation efficiency and bioavailability (>90%) of the active agent
- Minimal “burst” from the active agent
- Low levels of toxic agents used in formulation (excluding active agent)
- Process scalable to large (g-kg) quantities

In practice, it is very difficult to satisfy all of the aforementioned criteria. For example a particular surfactant may be used in order to obtain the optimum particle size, however the surfactant may remain at too high a level after purification to justify using it depending on its toxicity. Compromises usually have to be made in at least one of these conditions when preparing nanoparticles.

Common methods used in the preparation of nanoparticles include the creation of oil-in-water (O/W) and water-in-oil-in water (W/O/W) emulsions by using high speed homogenizers and/or probe-tip sonicators. The nanometer size organic droplets are stabilized by using large amounts (\geq amount of biodegradable polymer) of surfactant. The particles are formed after evaporation of the organic solvent or by using an in-liquid drying process (organic solvent extraction). Particles are collected and excess surfactant removed by ultrafiltration or ultracentrifugation. Potential problems include chemical and/or physical degradation of the active agent induced by the high stress forces that accompany homogenizers and sonicators, or through destructive interactions with the organic solvent (e.g. denaturation of proteins). Another problem with using high stress devices in the production of nanoparticles is the relatively small quantity of particles that are produced. These devices only work with small volumes (typically <5ml) that severely limit the quantity of PLAGA that can be used in the formulation. Nonetheless, there are a number of references to nanoparticle formulations that exhibit most of the desired characteristics mentioned earlier. The notable exception is usually quantity of particles prepared.

In order to avoid the use of devices that induce great physical stress on emulsion components, another method involves the precipitation of the nanoparticles through the use of an organic solvent that is entirely miscible with water. Acetone is a reasonable solvent for lower molecular weight PLAGA, is miscible with water in all proportions, and has been demonstrated as an effective solvent for the production of nanoparticles. Upon mixing of the organic and aqueous phases, the PLAGA immediately precipitates to form the nanoparticles. Typically, it is not necessary to use surfactant in order to produce the nanometer-sized particles when utilizing acetone. This technique also allows for the preparation of larger quantities of PLAGA nanoparticles since sonicators and homogenizers are not used in the process.

We have prepared formulations of PLAGA nanoparticles using several combinations of organic solvents and surfactants. Our methods involve brief sonication, and also allow for the production of gram quantities of nanoparticles if so desired.

Separation of the nanoparticles from the surfactant is also considered along with the ability to re-suspend the nanoparticles after freeze-drying. Comparisons of formulation yield, nanoparticle size, ease of surfactant removal (if desired), and ability to re-suspend the freeze-dried material are presented.

Challenges in Nanoparticle Preparation

One of the greatest challenges in preparing nanoparticles, as opposed to microparticles, is the removal of undesirable surfactant. For microparticles, it is a fairly simple procedure to filter, collect and wash the microparticles using a variety of filter papers in a short time period. However, purification of nanoparticles is a much more complicated procedure and one that can take a considerable amount of time. We have investigated both dialysis and cross-flow filtration as purification procedures during the first 12 months of this project. These methods have had limited success and specific results will be discussed later in this report. The most consistent result, unfortunately, was that for formulations where a significant amount of the surfactant had been removed, the formulations would aggregate during freeze-drying and not resuspend as individual nanoparticles. This was not an acceptable behavior so we investigated a number of different surfactants which could be used in nanoparticle preparation and where higher residual amounts of surfactant would be acceptable in the final formulation. Although nearly all investigators preparing nanoparticles from PLAGA use poly(vinyl alcohol) as the surfactant during nanoparticle preparation, we evaluated a number of surfactant and solvent combinations in order to develop a preparation method which would use the most benign materials while still giving particles in the desired size range, which can resuspend easily after freeze-drying and which show controlled drug delivery.

Results and Discussion

Nanoparticles Preparation Methods

Materials and Methods

Biodegradable PLAGA (65:35 lactide:glycolide, MW 33,000) and PLAGA-PEG (70:30 lactide:glycolide, inherent viscosity 0.72 dL/g, PEG molecular weight 5,000) were purchased from Birmingham polymers (Birmingham, AL). SSA-PEG (N-hydroxysuccinimidyl active ester of PEG succinamide, molecular weight 5,000) was purchased from Shearwater Polymers. The solvents dichloromethane (DCM), ethyl acetate (EA), and acetone (Ace), as well as the surfactants polyoxyethylene sorbitan monooleate (PS80), sodium dodecyl sulphate (SDS, >99%), sodium cholate (SC, 99%) and human serum albumin (HSA, fraction V 96-99%) were purchased from Sigma (St.

Louis, MO). The surfactant poly(vinyl alcohol) (MW 6000, 88% hydrolyzed) was purchased from Polysciences (Warrington, PA).

Briefly, we have determined that the best method for preparing resuspendable, biodegradable nanoparticles in our desired size range follows this procedure. First, 100 mg of PLAGA was dissolved in 3 ml of organic solvent in a 20 ml scintillation vial. If SSA-PEG was to be included, it was either added directly to the organic solvent or it was dissolved in a separate amount of methanol and then this solution was added to the polymer/solvent solution. To this solution was added 10 ml of surfactant solution (1.5 to 10 mg/ml) and the resulting emulsion was very briefly shaken by hand before being immersed in a bath-type sonicator (Aquasonic, model 50D) operating at 45W for 1-minute. The emulsion was then transferred to a vacuum Erlenmeyer flask (150 ml) with an additional 10 ml of water used as a rinse. The emulsion was stirred at 400 RPM while under a modest vacuum (\approx 100 mm Hg, with bleeding) for 30-45 minutes to remove residual solvent. After the organic solvent was completely removed, a small volume of the emulsion was removed (while stirring continued) for sizing using a Coulter Nanosizer calibrated with 200 nm latex spheres (Polysciences, Warrington, PA). The emulsion was then freeze-dried and the nanoparticles stored for later use. Nanoparticles prepared from PLAGA-PEG were prepared in a similar fashion, although additional SSA-PEG was not added in these cases. A summary of all nanoparticle formulations prepared and their specific formulation parameters may be found in Appendix A of this report.

Nanoparticle Analysis

If it was desired to remove surfactant, the emulsion was either dialyzed against pure water using membranes of 15 or 50K molecular weight cut-off or filtered by cross-flow filtration with a Spectrum Microgon MiniKros sampler system using a 50 nm cutoff cross-flow module. If acetone was used as the organic phase solvent, the mixing of aqueous and organic phases was done without sonicating.

Assays for PVA were carried out using published methods involving complexation with iodine[1]. A similar qualitative procedure was developed for SC. Briefly, the filtrate solution (either from simple dialysis or CFF) was boiled down to a volume of 2-3 ml and made acidic (pH=2) with a hydrochloric acid solution. To this solution, one ml of a saturated iodine solution was added and the presence of a blue precipitate indicated that SC was in the solution at a concentration $> 100 \mu\text{g/ml}$. We were unable to find published assays for the other surfactants tested, therefore, when appropriate mass balance studies were used as a qualitative measure of the effectiveness of surfactant removal from a formulation.

Larger quantities (gram) of nanoparticles could be obtained by preparing multiple suspensions in 20 ml scintillation vials as discussed earlier and then adding them all together before the final solvent removal stage.

Nanoparticle sizes for the most successful drug-free formulations prepared from different solvents and surfactants are summarized in Table 1. A summary of all formulations prepared is given in Appendix A of this report. The smallest particles were obtained using the surfactant/solvent combination of SDS and ethyl acetate (65 nm) and the largest particles were obtained using PVA and dichloromethane (466 nm). Polydispersity index (PI) as measured by the Nanosizer ranges from 0-9 with PI increasing with increasing index number (PI=0-1 monodisperse, PI=8-9 ratio of largest to smallest particle 4-5). Typical values for the PI with our formulations are 3-4, however, they may be as high as 9 depending on the formulation. In formulations where large aggregates were observed, the aggregates were allowed to settle (\approx 20 min) before the sample was sized. Allowing the aggregates to settle brought the measured particle size under 1 micron and the PI below 9. Formulations observed visually to have a significant amount of aggregate formation are indicated in Table 1. Those sizes that are marked with an asterisk slowed significant aggregation, resulting in low yields of particles in the desired size range. When DCM was used as the organic phase solvent, nearly every formulation resulted in significant aggregation, the only exception being the formulation using SDS as the surfactant. Ethyl acetate fared slightly better than DCM with regards to aggregate formation, however, acetone worked best, only forming aggregates when no surfactant was present in the formulation.

Table 1. Average Particle Size of Formulations Prepared using Various Solvents and Surfactants

[in nanometers as measured by Coulter Nanosizer (polydispersity index)]

	PVA (15 mg)	PS80 (15 mg)	SDS (15 mg)	SC (50 mg)	HSA (100 mg)	None
DCM	*466 (5)	*426 (6)	228 (4)	*393 (5)	*309 (5)	...
EA	*257 (2)	274 (3)	65 (4)	*130 (3)	*230 (3)	...
Ace	185 (2)	179 (3)	285 (4)	200 (3)	175 (3)	*261 (3)

Attempts at removing surfactant immediately after solvent evaporation, for the most part failed. Cross-flow filtration removed lower molecular weight surfactants (SDS and SC) within a couple of hours, however, removing these surfactants resulted in immediate aggregation of all particles. PVA and PS80 could be removed over the course of a few hours, however, it has been shown that PVA cannot be completely removed from the nanoparticles using this technique and can be present at levels as high as 10% by mass. In addition, recovery of nanoparticles becomes increasing inefficient as cross-flow filtration time increases. Recovery is typically 10% or less after 5-6 hours of cross-flow filtration of PVA nanoparticle formulations. In order to test the cross-flow filtration system as a source of the aggregation, blank nanoparticles created from an acetone nano-precipitation were pre-filtered to remove large aggregates and then placed as a suspension in the cross-flow filtration instrumentation. After one hour of CFF, the sample suspension was cleared as all of the particles aggregated into large (mm-sized) particles.

Equilibrium dialysis was also attempted with the lower MW surfactants SDS (MW=288 g/mol) and SC (MW=431 g/mol) using a 15K and 50K molecular weight cut-off membranes. After 24 hours and several external bath replacements, significant amounts of surfactant remained in the dialysis tubing (as determined by mass balance). In addition, aggregates began to form and settle inside the dialysis membrane.

If particle suspensions were freeze-dried without any attempt at removing the surfactant, then mass recovery yields were high (nearly 100%), however, many formulations did not re-suspend as submicron particles but instead only as very large (mm-size) aggregates. The two exceptions were those formulations prepared using either PVA or HSA as the surfactant. Formulations using PVA or HSA typically re-suspend to the same size and PI after freeze drying when using the amounts indicated in Table 1.

Discussion

Particle size and surfactant/solvent combinations

One goal of this research was to identify a surfactant suitable for use in controlled release formulations that satisfied the previously mentioned criteria. Of primary concern was to develop a simple formulation that resulted in the creation of submicron particles with a high efficiency and zero or very low levels of toxic materials present (other than encapsulated drug).

There are several factors that effect particle size with two of the most significant being PLAGA concentration in the organic phase (viscosity) and surfactant concentration in the aqueous phase. Reducing the former minimizes particle size, but also reduces the amount of material recovered. We decided to fix the PLAGA concentration at 33.3 mg/ml and the organic solvent volume at 3 ml so that we could obtain an appreciable amount of nanoparticles without using volumes too large for our sonicator to effectively create an emulsion. The surfactant concentration was then minimized to the mass needed in order for the most effective surfactant to create a stable emulsion without any aggregation. Unless the surfactant proved to be unable to stabilize the emulsion to any degree, the minimal amount of surfactant (15 mg) was used for every formulation. Sodium cholate and HSA both proved to be completely ineffective with only 15 mg and therefore their amounts were increased accordingly. There are two factors to consider when analyzing the effectiveness of a particular surfactant. First, the ability of the surfactant to stabilize a nanoparticle emulsion when first created and second, the ability of the surfactant to stabilize the nanoparticles after drying such that they will re-suspend in aqueous solutions.

Of the surfactants tested, it is clear that SDS works best with regard to the efficiency of preparing submicron particles (minimal amount of surfactant (15 mg) with DCM). With DCM and EA as the organic phase solvents, using SDS as the surfactant also resulted in the smallest average particle size (228 and 65 nm, respectively). Unlike

the other surfactants tested, SDS has a long hydrophobic region (12 carbon chain) and a relatively small hydrophilic sulphate anion at the end of the hydrophobic chain. PVA, PS80 and HSA contain hydrophobic and hydrophilic regions heterogeneously dispersed along the length of the molecule and therefore are not as isolated as they are for SDS. The structure of SC is such that its hydrophilic (hydroxyl and carboxylic groups) and hydrophobic groups are on opposite sides of a steroid nucleus, but does not contain any long hydrophobic chain similar to SDS. It is likely that the long hydrophobic chain of an SDS molecule will bind to the surface of an organic phase droplet with a significantly higher binding constant relative to the other surfactants, thus making SDS more efficient at lowering the interfacial tension, resulting in smaller particles and an overall more efficient process. The difference in average particle size between DCM and EA solvents when using SDS as the surfactant is again attributed to the lower the interfacial tension achieved with the slightly water soluble EA.

Surfactants other than SDS displayed at least some difficulty in stabilizing a nano-emulsion using our formulation methods. With DCM, none of the other surfactants were able to prevent aggregation of PLAGA. This is attributed to the fact that DCM has low miscibility with water which creates the need for a highly efficient surfactant to stabilize an emulsion. When using the slightly water miscible EA, only PS80 was able to prevent significant aggregation. The ability (or lack thereof) of these surfactants to stabilize these emulsions under described conditions is attributed to their relative ability to lower the interfacial tension between aqueous and organic phases when the emulsion is first created. This in turn is reflective of the ability of the hydrophobic portion of the surfactant molecule to bind to the oil droplet and the hydrophilic portion of the surfactant molecule to remain saturated in the water phase. The rank order of surfactant stabilization efficiency is then SDS>PS80>PVA>SC>HSA. With SC and HSA being rated as the least efficient because larger amounts are required to prepare a partial nano-emulsion.

When acetone is the organic phase solvent, there is no creation of an emulsion, but rather the PLAGA immediately precipitates as submicron particles[2-5]. It has already been shown that the presence of surfactant in the aqueous phase is not necessary to create submicron particles[3, 4]. However, it is clear from our results that surfactant may aid in reducing aggregation of the particles once the nano-precipitates are formed. PVA and PS80 appear to be the most suitable surfactants at reducing aggregation of the particles suspended immediately after formation (smallest average particle size). Interestingly, SDS is the worst stabilizer studied when used with acetone. Average particle sizes from the SDS/acetone combination are about the same as that when no surfactant is present in the aqueous phase. These results are indicative of the relative binding affinity of the surfactant to the PLAGA nanoparticle surface while fully hydrated.

Surfactant removal/particle aggregation

SDS can be removed from the formulation either by simple dialysis or cross-flow filtration techniques. Dialysis was found to be extremely inefficient, taking 24 hours to remove $\approx 90\%$ (mass balance) of the material using 50,000 MWCO membrane. A previous study attempting to quantify residual SDS on the surface of the particles purified by dialysis showed substantial amounts of SDS remaining in the formulation. However, the authors used dialysis membrane with a molecular weight cut-off of 6000-8000 for 24 hours[6]. The molecular weight of the SDS anion is 265 g/mole and its critical micelle concentration is 0.23mg/ml at 20°C. Because removal of SDS through a dialysis membrane will depend on the micelle \rightleftharpoons monomer equilibrium constant, it is likely that the efficient removal of SDS from any formulation through simple dialysis will require a membrane with a MWCO significantly higher than 50,000. Considering the relatively high molecular weights of surfactants and the relatively short degradation times of submicron PLAGA particles, dialysis is not a recommended technique for surfactant removal from formulations.

Cross-flow filtration is a considerably more efficient process than simple dialysis as it allows for the use of much higher MWCO membranes, the membrane surface area to sample volume ratios are much higher and the internal solution is pumped across the membrane at higher pressure. Using a 50 nm cutoff membrane was found to be extremely efficient at removing SDS from the formulation (≈ 1 hour to remove $>98\%$, mass balance), however as the SDS was removed the particles aggregated to very large sizes (all material $>1\mu\text{m}$). Once the surfactant is removed, the hydrophobic surface of the nanoparticles results in the aggregation and renders the particles unusable for intravenous injection as it is not possible to re-suspend these particles in an aqueous environment. Interestingly, even if the SDS is left in the formulation without any attempts to remove it, the nanoparticles will not re-suspend after being freeze-dried. Possibly, the SDS does not remain bound to the nanoparticle surface upon freeze-drying allowing the hydrophobic particles to aggregate. Identical results were obtained for PS80 and SC. PVA was found to be much more difficult to remove by cross-flow filtration as a result of its higher molecular weight (6000). However, more than 90% of the PVA could be removed within 6-7 hours of cross-flow filtration. Due to the significantly higher molecular weight of HSA (68000), we did not attempt to remove it by cross-flow filtration as the filtration times would have been much too long. Another method commonly used for surfactant removal is ultracentrifugation with subsequent washings using distilled water. Disadvantages of this technique include very small sample volumes (1-2ml) and extreme physical stresses on the particles that as yet cause an undetermined amount of damage. It is also clear that this technique removes only solvated surfactant and not that bound to the particle surface, otherwise the particles would not re-suspend.

In order for particles to re-suspend after freeze-drying, their surfaces must be rendered hydrophilic by a suitable surfactant. The surfactant must also have a relatively high affinity for the particle surface such that it will not desorb during the freeze-drying process and leave the particle surface hydrophobic. PVA and HSA are both known to bind to PLAGA surfaces with a high affinity[3, 7-9]. The affinity of the other surfactants tested for PLAGA surfaces were unknown, but are obviously insufficient at rendering the PLAGA surface hydrophilic. One other surfactant (Poloxamer-188/ Pluronic F68)

demonstrates a similar effect (desorbs) when freeze-dried with PLAGA nanoparticles[10].

Re-suspension/Aggregation

Another requirement of any formulation is its stability under long-term storage conditions. Therefore, it will be necessary to freeze-dry the nanoparticles to prevent hydrolytic degradation. Consequently, the formulation must re-suspend to submicron sizes in diluent (i.e. saline) after being freeze-dried.

It is interesting that all formulations result in the formation of nanoparticles suspensions before freeze-drying, including the formulation involving acetone without the use of any surfactant. This indicates the 65:35 PLAGA nanoparticle surface is hydrophilic enough such that the nanoparticles will remain suspended immediately after formation. Thus, it is the freeze-drying process that renders the particle surface hydrophobic.

Naked PLAGA particles will not re-suspend due to the hydrophobic nature of the polymer. However, if the particles can be protected from aggregating while being freeze-dried and/or their surfaces rendered hydrophilic, then re-suspension of the dried nanoparticles should be relatively straightforward. Since the surfactant stabilizers should be well suited for rendering particle surfaces hydrophilic, we freeze-dried our formulations without removing any surfactant. Of the surfactants tested, only PVA and HSA allowed for the re-suspension of the formulation to submicron sizes. It is known that PVA and HSA have a high affinity for PLAGA so it is not surprising that formulations containing these surfactants performed well in the re-suspension tests. However, it is surprising that SDS, SC, and PS80 do so poorly. None of the formulations that utilize these surfactants produce nanoparticles that re-suspend in water or saline. Clearly the nanoparticle surface is not rendered hydrophilic by these surfactants such that they will re-suspend in aqueous solutions. PS80 and SC were less efficient with regards to nanoparticle efficiency and size so it may be that these surfactants do not have as high an affinity for the PLAGA substrate. SDS on the other hand was the most efficient surfactant tested, in regard to particle size and efficiency. Therefore, it is surprising that these formulations did not re-suspend in aqueous solution. The long hydrophobic tail present on the SDS molecule should result in a high binding constant to the PLAGA substrate. However, the relatively small hydrophilic portion of the SDS molecule (with respect to the rest of the molecule) evidently does not provide sufficient coverage to prevent particle aggregation thus affecting its ability to re-suspend.

Optimal Formulations

PVA has by far been the most commonly used surfactant in PLAGA micro/nanoparticle formulations. With our methods, we found it to be adequate but not exceptional for formulating submicron PLAGA particles. With DCM and EA, significant

particle aggregation occurs, although the average size is reasonably small once the aggregates have been allowed to settle. Most of the literature concerning PVA and nanoparticles cite the use of ultracentrifugation for removing the PVA from the formulation, however, very few actually assay for residual PVA. The one consistent exception concerns the work by Alleman et al, that showed the presence of residual PVA on 200 nm PLAGA particles as high as 10% by mass[1]. PVA is of questionable toxicity. Studies in rats/mice have shown that PVA tends to accumulate in the lungs, kidneys and liver when administered intravenously, although no long-term toxic effects are known[11, 12].

HSA is a protein of MW 68,000 and thus is very difficult to remove from formulations using any type of dialysis technique. Centrifugation could remove free HSA in solution, but then the particles would not re-suspend after freeze-drying. Our initial reasoning behind using HSA as a surfactant was so that we would have a non-toxic biomaterial that would not have to be removed from the formulation. Thus, eliminating a purification step from the process that will result in higher yields and simpler techniques. HSA is routinely used as a principal component of injectable protein formulations in intramuscular and other types of injection. Therefore, its inclusion in these nanoparticle formulations should not have any detrimental biological effects.

It has been shown that albumin will readily bind to nanoparticle surfaces, increasing the diameter of a 200 nm particle by \approx 5% [3]. Free HSA will establish an equilibrium with surface-bound material, thus it is important that none of it be removed. We found that 100 mg was the minimum amount of HSA needed in our formulations to ensure good re-suspendability of the nanoparticles after freeze-drying.

Of the three solvents tested, only DCM did not work well for the production of submicron particles using our methods. Low efficiencies and large average particle size were typical with DCM formulations. With the method employed, we were unable to provide sufficient energy to the emulsion in order to create a submicron particles efficiently. This is likely the result of DCM's extremely low solubility in water creating significantly higher interfacial tension as compared to the slightly soluble EA and completely soluble acetone. The method could have been altered to use a more dilute PLAGA solution as well as more surfactant in order to produce smaller particles and an overall more efficient process. However, to alter the method in this way would have meant fewer particles and a higher surfactant concentration in the final freeze-dried product, both undesirable consequences.

When EA was used as the organic phase solvent, we obtained mixed results. Particle aggregation using EA was less severe than with DCM but still presented problems with 3 of the 5 surfactants tested (Table 1). With respect to particle size, EA faired better than DCM but not as well as acetone in terms of smaller particle diameters. EA is slightly soluble in water, therefore, these results are consistent with a lower interfacial tension between aqueous and organic phases in the emulsion. What is of particular interest is the dramatic reduction in particle sizes when using the anionic surfactants in combination with EA. Currently, we have no data to suggest why the use

of SDS and SC as surfactants in combination with EA as the organic phase solvent result in significantly smaller particles. We speculate that the handedness (separation of hydrophilic and hydrophobic regions of the molecule) of SDS and SC creates an optimal situation for both binding to the surface of the organic droplet with its hydrophobic component and binding to water with its hydrophilic component, thus allowing these molecules to be much more efficient at reducing the interfacial tension between the two phases.

Acetone is completely miscible with water, therefore stable emulsions between organic and aqueous phases cannot be created under our conditions. The nanoparticles are formed immediately upon mixing the two phase through by precipitation of the PLAGA [2]. This can be highly beneficial in terms of drug encapsulation, nanoparticle efficiency, and general ease of the preparation. However, if the suspension is to be freeze-dried, surfactant (PVA or HSA or anything that will render particle surface hydrophilic) must be present in the solution.

Based upon these results, the optimal formulations, from a standpoint of particle size and resuspendability, are those prepared using human serum albumin as the surfactant and acetone as the organic solvent.

Drug Delivery Studies

Materials and Methods

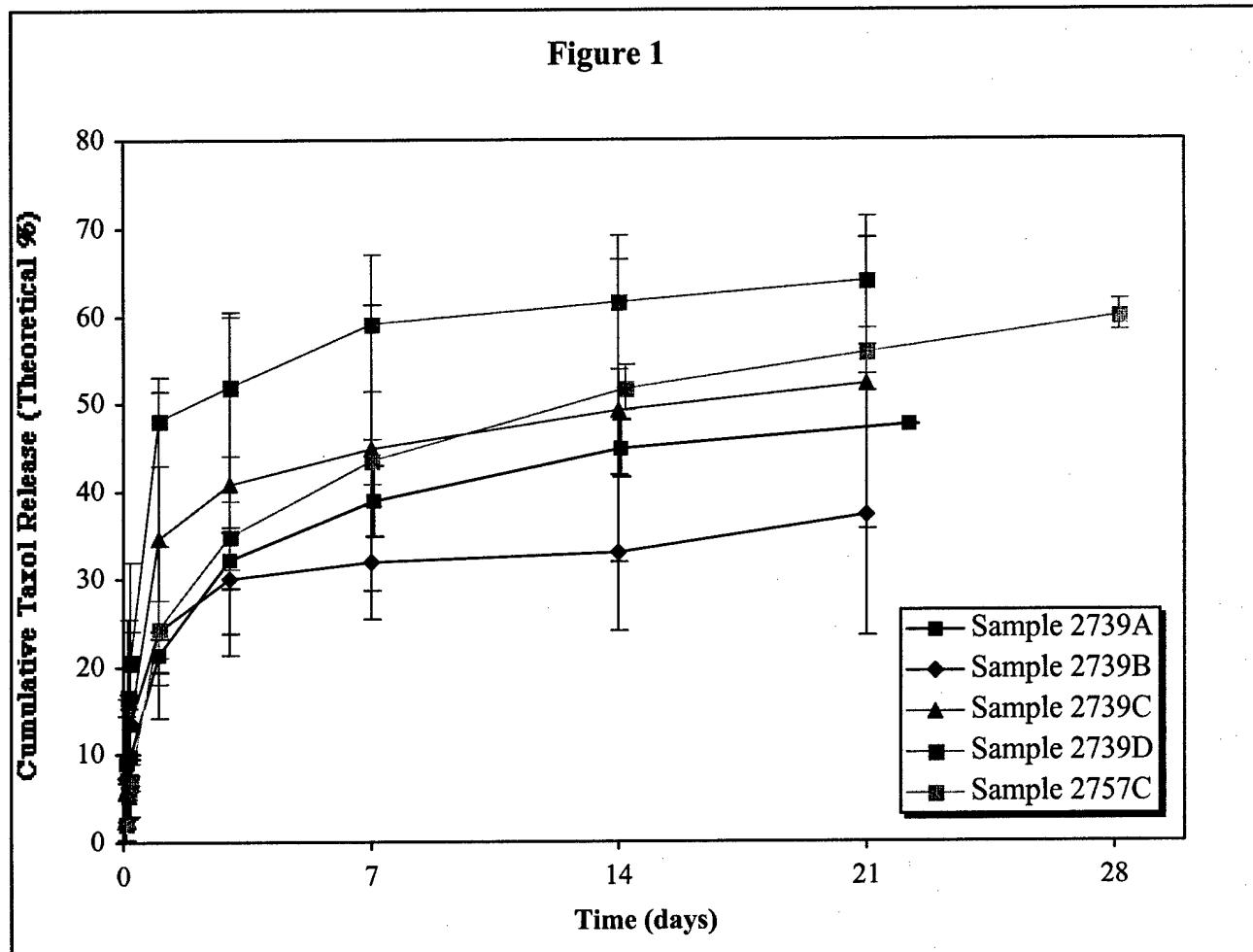
At this time, *in vitro* drug delivery studies have been conducted for formulations containing paclitaxel (Taxol). In these studies, dialysis cells with 1ml-capacity cavities (Bel-Art Products, Pequannock, NJ) are fitted with Spectra/Por® Biotech cellulose ester dialysis membranes (Spectrum, Laguna Hills, CA). Particles (20-50 mg) are suspended in 1 ml of a 10:1 mixture of saline and cremaphor and injected into one cavity (donor side). The cremaphor is present to assist in the solubility of the taxol. Fresh saline/cremaphor is injected into the other cavity (recipient). The cells are placed in a heated, shaking water bath (37°C). At predetermined times, the recipient solution is removed and completely replaced with fresh saline.

Samples are filtered through 0.45 μ m syringe filters and the absorption of each is measured by HPLC. The HPLC assay utilizes a Phenomenex Curosil-8 5 μ column specifically designed for separation of taxol. The HPLC system includes Waters autosampler, pumps, gradient controller and UV-Vis detector. The sample injection volume was 15 μ l with a flow rate of 1.0 ml/min and a linear gradient from 60:40 to 25:75 acetonitrile:water over 15 minutes. Detection is performed at 254 nm. All studies are performed in triplicate. The HPLC data is analyzed using a Macintosh computer and MacChrom chromatography data analysis software.

Discussion

The cumulative taxol release from five representative formulations is shown in

Figure 1. All release studies were carried out from 21-28 days and all studies were done in triplicate. The error bars shown are the standard deviation of the data. The optimal formulation will show the most linear release profile as well as the least deviation in behavior among samples.



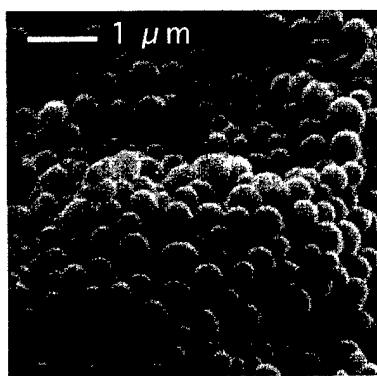
All of these formulation showed a "burst" of release within the first day of less than 50%, which is good performance for nanoparticle formulations which can show a much higher initial release rate. These results are based on release from approximately 10 mg of nanoparticles for each *in vitro* sample tested. The best performing formulation to date (2757C) only showed 24% release within the first day and 43% in the first week. This particular formulation, which was prepared using acetone as the organic solvent and albumin as the surfactant, has shown the best performance in terms of particle size, resuspendability and drug delivery. Therefore, some additional work will be done based on this basic formulation in order to establish a more constant rate of release for an even longer time period. Then these formulations, with varying amounts of taxol, will be the first to go into cell culture performance and targeting studies.

Inclusion of Targeting Antibodies in Formulations

In previous work done by Biogel Technology, subsequent to the submission of this proposal, we developed techniques to successfully bind fibrinogen to the PEG arms of nanoparticles prepared from a mixture of PLAGA and SSA-PEG. We evaluated a number of preparation techniques and found that the most successful method for such attachment was to add the agent to be attached to the stirring nanoparticle preparation solution one hour before the end of the procedure would be completed. The only requirement for this attachment to occur is that the pH of the aqueous solution be adjusted to 7-9. Under these conditions, attachment will occur within 30 minutes of stirring. As soon as the cell-based assays are in place at Indiana University, formulations will be prepared using this technique containing taxol having the monoclonal antibody (MAb) to HER-2/neu bound to their surface. Work is currently underway to modify the PLAGA-PEG so that similar attachment procedures may be used with it as well.

Nanoparticle Preparation

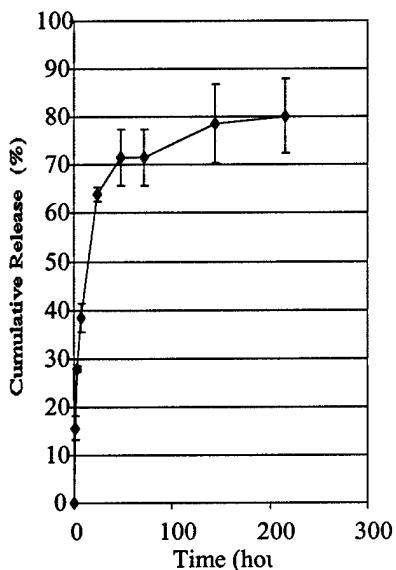
Biodegradable PLAGA (65:35 lactide:glycolide, MW 33,000) was purchased from Birmingham polymers (Birmingham, AL). Acetone, taxol and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Doxorubicin was provided by Pharmacia-Upjohn and the herceptin was provided by Indiana University School of Medicine. For the general nanoparticle preparation process, 100 mg of PLAGA was dissolved in 3 ml of acetone in a 20 ml scintillation vial. To this solution was added 10 ml of BSA solution (1.0 mg/ml) and the resulting emulsion was very briefly shaken by hand before being immersed in a bath-type sonicator (Aquasonic, model 50D) operating at 45W for 1-minute. The emulsion was then transferred to a vacuum Erlenmeyer (150 ml) with an additional 10 ml of water used as a rinse. The emulsion was stirred at 400 RPM while under a modest vacuum (~100 mm Hg, with bleeding) for 30-45 minutes to remove residual solvent. After the organic solvent was completely removed, a small volume of the emulsion was removed (while stirring continued) for sizing using a Coulter Nano-Sizer calibrated with 200 nm latex spheres (Polysciences, Warrington, PA). The solution was centrifuged and washed three times with progressively more dilute BSA solutions to remove unencapsulated drug and unbound antibody.



A scanning electron micrograph of PLAGA nanoparticles containing doxorubicin and with herceptin attached is shown here.

Drug Delivery

Release studies were carried out using equilibrium dialysis. Dialysis cells with 1 ml capacity on both the donor and receptor sides were prepared with approximately 50 mg of freeze-dried formulation material (surfactant and particles) suspended in 3 ml of buffered saline. The receptor side was separated from the suspension side by dialysis membrane of 50K molecular weight cutoff. Sample solutions were removed and replaced with fresh saline at each sampling time. The amount of taxol was measured using HPLC and the amount of doxorubicin was measured using UV-Vis Spectrophotometry. Release profiles were measured in triplicate and corrected for the volume of the suspension side of the dialysis cell. A representative release profile of doxorubicin from PLAGA nanoparticles with an average particle size of 312 nm is shown below.



Initial in vitro studies

Our initial goal has been to examine the ability of NP-MAb conjugates to bind to HER-2/neu-expressing cancer cells *in vitro* by flow cytometry. This was initially performed through the following experiment: freshly prepared nanoparticles containing doxorubicin were either conjugated to Herceptin monoclonal antibody or not, then mixed with breast cancer cells that are or are not positive for HER-2 cell surface expression. The results of multiple flow cytometric analyses suggested that this approach was not technically feasible, in that fluorescence intensity was similar for all tested cells.

The explanation for this failure presumably represented the early release of doxorubicin from nanoparticles, uptake by breast cancer cells, and fluorescence of the breast cancer cells. We subsequently prepared a total of 53 formulations using doxorubicin or epirubicin from nearly every PLGA available (Birmingham Polymers and

Alkermes). All but two formulations used 100 mg of PLGA, with 2 lots prepared at 1 gram of PLGA. The Alkermes PLGA lot 9007-394 gave the overall best performance based on encapsulation, drug burst, length of release, and total amount drug released. PLGA lot 9007-394 is acid-capped 50:50 PLGA with a MW of about 11 KDa.

PLGA lots from BPI tested were D97044 (65:35, 33000 MW), D97121 (65:35, 50000 MW) and D95061 (70:30 grafted to 5000 MW PEG). We also performed chemical modifications of D95061 and used the resultant polymer in a few formulations. Most of these formulations used acetone as the organic phase solvent and albumin as the aqueous phase surfactant. In general, formulations prepared from these polymers did not perform well. Specifically, the encapsulation efficiency and release profiles were the most disappointing. Encapsulation efficiencies were in the 10-30% range and the release profiles showed a larger burst and a total release time of only 24 hours.

PLGA lots from Alkermes consisted of acid-capped and ester-capped 50:50's with varying molecular weights. Using ester capped PLGA's from Alchermes resulted in similar results to the BPI polymers (lower encapsulation efficiency, shorter release, larger burst, less drug released etc.) The acid capped polymers performed extremely well with regard to encapsulation efficiency of epirubicin and doxorubicin (nearly 100% regardless of PLGA MW). However, the acid-capped PLGA with a MW of 11000 (9007-394) clearly performed the best in terms of nanoparticle yield, re-suspendabilty and total drug released in vitro. We varied the drug loadings between 2 and 10 % by mass using 9007-394. All these formulations outperformed the ester-capped and BPI np's, however, the nanoparticles with the lowest amount of drug had the best looking release in vitro profile in terms of burst and total amount of encapsulted drug released.

SSA-PEG and the antibody have been included in some formulations as well. The presence of SSA-PEG was verified using 1-gram formulations such that sufficient sample could be tested. Only those particles without doxorubicin or epirubicin could be assayed for SSA-PEG as the drug greatly interferes with the assay. An attempt to assay for residual antibody on the nanoparticles was inconclusive.

In Vitro Cell Line Analysis

Binding and internalization of the reformulated Nanoparticle: Monoclonal Antibody conjugates were now tested, comparing binding to HER-2 positive (BT-474) and HER-2 negative (MDA-MB-435) human breast cancer cell lines. As predicted, superior binding of NP:MAb conjugates to the HER-2 positive cell line was seen. This finding was confirmed by scanning electron microscopy, which revealed virtually no binding of the NP:MAb conjugate to MDA-MB-435 cells, and extensive cell membrane localization of nanoparticles.

Similarly, the in vitro effects of NP:MAb conjugates on cell proliferation was examined using the CellTiter 96 AQ assay (Promega Corp., Madison, WI). We compared IC50's for the NP:MAb conjugate either containing or lacking drug (paclitaxel

• or doxorubicin), drug alone, and nanoparticles and MAb alone. In vitro, the IC50's for naked drug (paclitaxel or doxorubicin) and NP:MAb conjugate containing drug were similar, and both were superior to MAb alone or NP:MAb conjugate not containing drug.

In Vivo Therapeutic Application

In vivo studies of the NP:MAb conjugate were now performed. NP:MAb conjugate containing doxorubicin and doxorubicin as a naked drug were injected via tail vein in 6-8 week old mice previously injected with 10^7 BT-474 human breast cancer cells (which express high levels of HER-2 and which have been used in prior studies of anti-HER-2 MAb localization in nude mice. Mice were killed (in triplicate) at various time points (1,2, 4, 8, 16, 24, 48, 72, 120, 196, and 360 h), and tissues (tumor, liver, spleen, serum, heart, lungs, kidney) removed to determine the specificity of delivery of the conjugate. Tissues were processed for assay of doxorubicin, and tumor:tissue ratios at varying time points determined. Assays of doxorubicin were performed by spectrofluorometric assay. To our very great disappointment, serum and intratumoral levels of doxorubicin were essentially identical at every time point tested, suggesting that in vivo (as opposed to in vitro) rapid release of chemotherapeutic drug from the nanoparticle was occurring.

Subsequent Studies

Reformulation of NP:MAb conjugates was now considered and attempted. These studies were in process at the completion of the funding period for the grant. Studies subsequent to the funding period involving NP's containing epirubicin have suggested that this agent may have characteristics superior to doxorubicin with regard to drug release. Subsequent funding has been obtained from Pharmacia to analyze NP's containing epirubicin as a means of delivering chemotherapy intraductally (per ductal lavage), an ongoing project.

Conclusions

During the course of the study significant progress has been made in understanding the nanoparticle preparation process, far beyond that which we had hoped we would be able to accomplish. We may consistently prepare formulations in an optimal size range less than 350 nm with excellent resuspendability. The formulations prepared with paclitaxel (Taxol) and subsequently with doxorubicin showed a range of drug delivery behaviors, with the optimal formulations in terms of nanoparticle preparation also showing the optimal drug delivery behavior *in vitro*. Nanoparticles of desired size ranges and resuspendabilities have also been prepared of PLAGA-PEG polymers

Similarly, initial *in vitro* analyses suggested that NP:MAb conjugates were effective at binding to cells containing HER-2 on the cell surface, and ineffective at binding to cells lacking HER-2 on the cell surface. It was therefore a major disappointment that subsequent *in vivo* analyses revealed that the NP:MAb conjugate was in no way superior to simple intravenous injected of doxorubicin (naked drug). It is unknown whether this failure represents a physicochemical problem specific to

doxorubicin *in vivo*, or whether it represents a more general problem. As described above, extensive formulation attempts were unsuccessful in developing a conjugate with the desired *in vivo* characteristics, so that the project must be considered a failure with regard to its primary endpoints.

As mentioned above, the failure of this project did not result in an end to our attempts to utilize this technology for *in vivo* research. A subsequent collaboration between Indiana University and Biogel is currently ongoing, studying the use of the nanoparticle system described above as a delivery system for administration of chemotherapy by ductal lavage. Initial results from this approach appear promising.

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Appendix A: Summary of Preparation Conditions for Biodegradable Nanoparticles

<u>Sample</u>	<u>PLAGA</u>	<u>Solvent</u>	<u>Surfactant and Amount</u>	<u>Aqueous Volume</u>	<u>Drug</u>	<u>ssa-PEG</u>
	(mg)		(mg)	(ml)	(mg)	(mg)
28-2	300	3 ml ethyl acetate	PVA, 100	10	-	-
28-5	300	3 ml ethyl acetate	PVA, 100	10	-	-
28-6	300	3 ml ethyl acetate	PS80, 200	10	-	-
28-7	300	3 ml ethyl acetate	SPAN 80, 200	10	-	-
28-8A	300	3 ml ethyl acetate	PVA, 30	10	-	-
28-8B	300	3 ml ethyl acetate	PVA, 50	10	-	-
28-10	300	3 ml ethyl acetate	PVA, 6.6	10	-	-
28-11	500	3 ml ethyl acetate	PVA, 20	10	-	-
28-12	500	3 ml ethyl acetate	PVA, 50	10	-	-
28-13	300	3 ml ethyl acetate	PVA, 100	10	-	-
28-14	300	3 ml ethyl acetate	PVA, 100	10	-	-
28-15	300	3 ml ethyl acetate	SDS, 100	10	-	-
28-16	300	3 ml ethyl acetate	PS80, 100	10	-	-
28-17	300	3 ml ethyl acetate	SDS, 50	10	-	-
28-18	300	3 ml ethyl acetate	SDS, 50	10	-	3
28-21A	100	3 ml ethyl acetate	SDS, 15	10	-	-
28-21B	100	3 ml ethyl acetate	SDS, 15	10	-	-
28-21C	100	3 ml ethyl acetate	SDS 15	10	-	-
28-22A	100	3 ml ethyl acetate	SDS, 15	10	-	-
28-22B	100	3 ml ethyl acetate	SDS, 15	10	-	-
28-22C	100	3 ml ethyl acetate	SDS 15	10	-	-
28-22D	100	3 ml ethyl acetate	SDS, 15	10	-	-
28-24	100	3 ml ethyl acetate	PVA, 15	10	-	-
28-25	100	3 ml ethyl acetate	SC, 40	10	-	-
28-27	100	4 ml ethyl acetate	SC, 50	10	-	25
28-31	100	3 ml ethyl acetate	SDS, 15	10	-	-
28-32A	100	3 ml dichloromethane	SC, 50	10	-	-
28-32B	100	3 ml dichloromethane	SDS, 15	10	-	-
28-32C	100	3 ml dichloromethane	PVA, 15	10	-	-
28-32D	100	3 ml dichloromethane	PS80, 15	10	-	-
28-33A	100	3 ml ethyl acetate	PS80, 15	10	-	-
28-33B	100	3 ml acetone	none	10	-	-
28-34A	100	3 ml acetone	PVA, 15	10	-	-
28-34B	100	3 ml acetone	PS80, 15	10	-	-
28-34C	100	3 ml acetone	SDS, 15	10	-	-

<u>Sample</u>	<u>PLAGA</u>	<u>Solvent</u>	<u>Surfactant and Amount</u>	<u>Aqueous Volume</u>	<u>Drug</u>	<u>ssa-PEG</u>
	(mg)		(mg)	(ml)	(mg)	(mg)
28-34D	100	3 ml acetone	SC, 50	10	-	-
28-35A	100	3 ml ethyl acetate	HSA, 100	10	-	-
28-35B	100	2 ml ethyl acetate, 1 ml acetone	HSA, 100	10	-	-
28-35C	100	1 ml ethyl acetate, 2 ml acetone	HSA, 100	10	-	-
28-35D	100	3 ml acetone	HSA, 100	10	-	-
28-36A	100	1 ml methanol, 2 ml acetone	HSA, 100	10	-	-
28-36B	100	0.5 ml methanol, 2.5 ml acetone	HSA, 100	10	-	-
28-50A	100	0.2 ml methanol, 3 ml acetone	HSA, 100	10	-	10
28-50B	100	0.2 ml methanol, 3 ml acetone	HSA, 100	10	-	5
28-50C	100	3 ml acetone	HSA, 100	10	-	-
28-52A	100	3 ml dichloromethane	HSA, 100	10	-	-
28-52B	100	3 ml ethyl acetate	HSA, 100	10	-	-
28-53	100	3 ml acetone	HSA, 100	10	-	-
28-54	100	0.5 ml methanol, 2.5 ml acetone	none	20	-	12.5
28-55	100	2 ml methanol, 4 ml acetone	none	50	-	50
28-57A	100	3 ml acetone	HSA, 10	10	-	-
28-57B	100	3 ml acetone	HSA, 25	10	-	-
28-57C	100	3 ml acetone	HSA, 50	10	-	-
28-59A	100	0.5 ml methanol, 2.5 ml acetone	HSA, 100	20	-	1
2727A	300	3 ml ethyl acetate	SDS, 50	10	-	30
2727B	300	3 ml ethyl acetate	SDS, 50	10	-	15
2727C	300	3 ml ethyl acetate	SDS, 50	10	-	3
2727D	300	3 ml ethyl acetate	SDS, 50	10	-	-
2727E	300	3 ml ethyl acetate	SDS, 50	10	-	3
2727F	300	3 ml ethyl acetate	SDS, 25	10	-	-
2727H	300	3 ml ethyl acetate	SDS, 50	10	-	30
2727J	300	3 ml ethyl acetate	SDS, 50	10	-	15
2731A	300	3 ml ethyl acetate	SDS, 50	10	-	-
2731B	300	3 ml ethyl acetate	SC, 50	10	-	-
2731C	300	3 ml ethyl acetate	SDS, 50	10	-	30
2731D	100	3 ml ethyl acetate	SC, 50	10	-	-
2735A	100*5	3 ml ethyl acetate	PVA, 50	10	-	-
2735B	100*5	3 ml ethyl acetate	PVA, 50	10	-	-

<u>Sample</u>	<u>PLAGA</u>	<u>Solvent</u>	<u>Surfactant and Amount</u>	<u>Aqueous Volume</u>	<u>Drug</u>	<u>ssa-PEG</u>
	(mg)		(mg)	(ml)	(mg)	(mg)
2735C	100*5	3 ml ethyl acetate	PVA, 30	10	-	-
2735D	100*5	3 ml ethyl acetate	PVA, 50	5	-	-
2735E	100*5	3 ml ethyl acetate	PVA, 50	5	-	-
2735F	100*5	3 ml ethyl acetate	PVA, 100	10	-	-
2735G	100*5	3 ml ethyl acetate	PVA, 35	7	-	-
2735H	100*5	3 ml ethyl acetate	PVA, 70	7	-	-
2739A	100	3 ml ethyl acetate	PVA, 50	10	10 mg taxol, 1 ml EA	-
2739B	100	3 ml ethyl acetate	PVA, 50	10	10 mg taxol, 1 ml EA	10
2739C	100	3 ml acetone	HSA, 100	10	10 mg taxol, 1 ml acetone	-
2739D	100	3 ml acetone	HSA, 100	10	10 mg taxol, 1 ml acetone	10
2741A	100	3 ml ethyl acetate	PVA, 50	10		1
2741B	100	3 ml ethyl acetate	PVA, 50	10		5
2741C	100	3 ml ethyl acetate	PVA, 50	10		10
2741D	100	3 ml acetone	HSA, 100	10	-	1
2741E	100	3 ml acetone	HSA, 100	10	-	5
2741F	100	3 ml acetone	HSA, 100	10	-	10
2757A	100	2.5 ml ethyl acetate	HSA, 100	10	10 mg taxol	10mg, 0.5 ml MeOH
2757B	100	3 ml ethyl acetate	HSA, 100	10	10 mg taxol	-
2757C	100	3 ml acetone total	HSA, 100	10	10 mg taxol, 1ml acetone	10mg, 0.5 ml MeOH
2757D	100	3 ml acetone total	HSA, 100	10	10 mg taxol, 1 ml acetone	-
2851B	100	3 ml acetone	HSA, 100	10	10 mg taxol, 200 μ l MeOH	-

<u>Sample</u>	<u>PLAGA</u> (mg)	<u>Solvent</u>	<u>Surfactant and Amount</u> (mg)	<u>Aqueous Volume</u> (ml)	<u>Drug</u> (mg)	<u>ssa-PEG</u> (mg)
2757G	100 (PLAGA-g- PEG, 70:30)	3 ml acetone	HSA, 100	10	-	-
2768B	100 (PLAGA-g- PEG, 70:30)	3 ml acetone	PVA, 15	10	-	-
2768C	100 (PLAGA-g- PEG, 70:30)	3 ml ethyl acetate	PVA, 15	10	-	-
2768D	100 (PLAGA-g- PEG, 70:30)	3 ml ethyl acetate	HSA, 100	10	-	-

Abbreviations:

EA: ethyl acetate

HAS: human serum albumin

MeOH: methanol

PS80: polysorbate 80

PVA: poly(vinyl alcohol)

SC: sodium cholate

SDS: sodium dodecyl sulfate

SPAN 80: sorbitan monooleate

Appendix B: Summary of Sizing Analysis for Biodegradable Nanoparticles

Sample	Average Particle Diameter Before Drying (nm); Polydispersity	Average Particle Diameter After Drying (nm); Polydispersity	Purification and Drying Treatment
28-2	185; 3	-	Freeze dried only
28-5	210; 2	600 (9)	Freeze dried only
28-6	175; 3	>3000*	Cross-flow filtered, freeze dried
28-7	360; 3	>3000*	Cross-flow filtered, freeze dried
28-8A	314; 3	411; 5	Freeze dried only
28-8B	246; 2	-	Not dried
28-10	485; 4	>3000*	Freeze dried only
28-11	351 (7)	>3000*	Not dried
28-12	332; 5	>3000*	Freeze dried only
28-13	288; 4	700 (9)	Not dried
28-14	290; 4	-	Not dried
28-15	64; 4	>3000*	Dialyzed, freeze dried
28-16	168; 2	>3000*	Dialyzed, freeze dried
28-17	64; 3	>3000*	Dialyzed, freeze dried
28-18	73; 4	>3000*	Dialyzed, freeze dried
28-21A	63; 4	>3000*	Dialyzed, freeze dried
28-21B	74; 4	>3000*	Dialyzed, freeze dried
28-21C	67; 3	>3000*	Dialyzed, freeze dried
28-22A	72; 4	>3000*	Freeze dried only
28-22B	67; 3	>3000*	Freeze dried only
28-22C	59; 3	>3000*	Freeze dried only
28-22D	56; 4	>3000*	Freeze dried only
28-24	257; 2	252; 4	Freeze dried only
28-25	102; 3	>3000*	Freeze dried only
28-27	137; 3	>3000*	Freeze dried only
28-31	104; 3	>3000*	Dialyzed, freeze dried
28-32A	393; 5	>3000*	Freeze dried only
28-32B	228; 4	>3000*	Freeze dried only
28-32C	466; 5	>3000*	Freeze dried only
28-32D	426; 6	>3000*	Freeze dried only
28-33A	274; 3	>3000*	Freeze dried only
28-33B	261; 3	>3000*	Freeze dried only
28-34A	185; 2	-	Freeze dried only
28-34B	179; 3	>3000*	Freeze dried only
28-34C	285; 4	>3000*	Freeze dried only
28-34D	200; 3	>3000*	Freeze dried only
28-35A	277; 3	270; 4	Freeze dried only
28-35B	258; 4	308; 3	Freeze dried only

28-35C 290; 5 301; 6 Freeze dried only

Sample	Average Particle Diameter Before Drying (nm); Polydispersity	Average Particle Diameter After Drying (nm); Polydispersity	Purification and Drying Treatment
28-35D	189; 3	184; 3	Freeze dried only
28-36A	164; 2	185; 2	Freeze dried only
28-36B	171; 2	183; 2	Freeze dried only
28-50A	193; 2	300; 4	Freeze dried only
28-50B	192; 3	198; 2	Freeze dried only
28-50C	191; 3	201; 3	Freeze dried only
28-52A	309; 5	293; 5	Freeze dried only
28-52B	230; 3	231; 5	Freeze dried only
28-53	188; 2	210; 3	Freeze dried only
28-54	197; 3	>3000*	Freeze dried only
28-55	132; 2	>3000*	Freeze dried only
28-57A	168; 3	>3000*	Freeze dried only
28-57B	165; 3	>3000*	Freeze dried only
28-57C	157; 3	>3000*	Freeze dried only
28-59A	160; 3	196; 4	Freeze dried only
28-59B	162; 6	215; 4	Freeze dried only
28-60	156; 3	445 (8)	Freeze dried only
28-61	2330 (9)	>3000*	Freeze dried only
28-66	113; 4	201; 5	Freeze dried only
28-75	163; 3		Freeze dried only
2727A	151; 3.7	> 3000*	Dialyzed, freeze dried
2727B	248; 5.3	>3000*	Dialyzed, freeze dried
2727C	>3000*	>3000*	Dialyzed, freeze dried
2727D	190; 5	>3000*	Dialyzed, freeze dried
2727E	231; 6	>3000*	Dialyzed, freeze dried
2727F	182; 3	>3000*	Dialyzed, freeze dried
2727G	166; 3	>3000*	Dialyzed, freeze dried
2727H	-	>3000*	Cross-flow filtered, freeze dried
2727J	-	>3000*	Cross-flow filtered, freeze dried
2731A	239 ; 4	>3000*	Freeze dried only
2731B	243 ; 3.3	>3000*	Freeze dried only
2731C	259 ; 4.3	>3000*	Dialyzed, freeze dried
2731D	197 ; 3.7	>3000*	Dialyzed, freeze dried
2735A	241 ; 4.3	280 ; 3.7	Cross-flow filtered, freeze dried
2735B	-	242 ; 3.3	Cross-flow filtered, freeze dried
2735C	-	585.6 ; 8	Cross-flow filtered, freeze dried
2735D	-	593 ; 7.7	Cross-flow filtered, freeze dried
2735E	-	640 ; 8.3	Cross-flow filtered, freeze dried
2735F	-	282.3 ; 7	Cross-flow filtered, freeze dried
2735G	-	243.7 ; 4.3	Cross-flow filtered, freeze dried
2735H	-	213; 3.7	Cross-flow filtered, freeze dried

2739A

219 ; 2.7 Freeze dried only

Sample	Average Particle Diameter Before Drying (nm); Polydispersity	Average Particle Diameter After Drying (nm); Polydispersity	Purification and Drying Treatment
2739B	215 ; 4	>3000*	Freeze dried only
2739C	310 ; 8	583.7 ; 9	Freeze dried only
2739D	259 ; 6.3	>3000*	Freeze dried only
2741A	-	223 ; 4	Freeze dried only
2741B	231 ; 2.3	261 ; 4.3	Freeze dried only
2741C	235 ; 2.7	>3000*	Freeze dried only
2741D	958 ; 8	1403 ; 7	Freeze dried only
2741E	369 ; 3.3	>3000*	Freeze dried only
2741F	183 ; 3	270 ; 5.7	Freeze dried only
2757A	>3000*	>3000*	Freeze dried only
2757B	>3000*	>3000*	Freeze dried only
2757C	238; 6.3	283; 6.3	Freeze dried only
2757D	231; 6.3	243; 6	Freeze dried only
2851B	250; 6.5	-	Freeze dried only
2757G	200; 5	618;7	Freeze dried only
2768B	232; 6.3		Freeze dried only
2768C	267; 6.3		Freeze dried only
2768D	250; 5.3	561; 6.3	Freeze dried only

* Nanosizer indicated that some of the particles were larger than 3000 nm and could not calculate an average size. Those samples where no size is given were not tested.

Mutation of the 18-mer sequence results in abrogation of RNP complex formation in the induced state.

Plasmid Construction—The four pairs of oligodeoxynucleotides (synthesized by Invitrogen as shown below) were respectively subcloned into the pSPT 18 vector which was linearized with EcoR I and Hind III to construct pYSN, pYSC1, pYSC2 and pYSC12.

N: 5'-p-AATT^CGCTCCATTCCC^CACTCC^CTGA-3'
3'-GCGAGCTAAGGGT^GAGGGACTTCGA-p-5'
C1: 5'-p-AATT^CGCTCCATTCCC^CACTACCTGA-3'
3'-GCGAGCTAAGGGT^GATGGACTTCGA-p-5'
C2: 5'-p-AATT^CGCTACATTCCC^CACTCC^CTGA-3'
3'-GCGATCTAAGGGT^GAGGGACTTCGA-p-5'
C1+2: 5'-p-AATT^CGCTACATTCCC^CACTACCTGA-3'
3'-GCGATCTAAGGGT^GATGGACTTCGA-p-5'

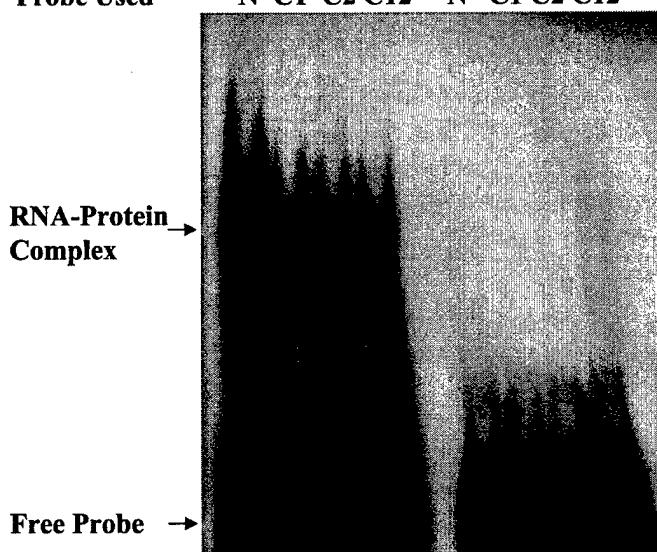
**The Sequences of Four Pairs of Oligodeoxynucleotides
for Generating 18-mer Sequences and Its Mutants**

Preparation of 18-mer RNA and Its Mutants—The four plasmid DNAs were linearized with Hind III and transcribed by T7 RNA polymerase respectively. Briefly, in vitro transcription was carried out at 37°C for 1 hour in the presence of 50 µCi of [α -³²P]-UTP and 0.5 mM of other three unlabeled nucleotides by T7 RNA polymerase. After transcription, template DNAs were digested with DNase I (RNase-free) and RNA transcripts were purified on NucTrap Push columns (Stratagene, La Jolla, CA).

RNA-Protein Binding and Gel-Shift Assay—Binding reactions were carried out with 40 µg cytosolic extracts from HeLa IU₁-HF cells and 5 X 10⁴ cpm of ³²P-labeled 18-mer or its mutants respectively in 10 mM Hepes, pH 7.6, containing 3 mM MgCl₂, 40 mM KCl, 5% glycerol, 600 ng/µl yeast tRNA, 1mM DTT in a final volume of 15 µl. After incubation at room temperature for 30 min, 20 units of RNase T1 and 1 µl of heparin solution (100 mg /ml) were added and incubation was continued for another 30 min. Electrophoresis of RNA-Protein complexes was carried out using 6% native PAGE (60:1) and dried gel were autoradiographed at -80°C

Result:

Cell Extracts (µg)	40	40	40	40	0	0	0	0
Probe Used	N	C1	C2	C12	N	C1	C2	C12



The Demonstration of a Supershift of RNP Complex Formation Induced in Response to High cellular Levels of Homocysteine Using Anti-hnRNP E1 Antibody

Preparation of Cytosonic Protein Extracts (S-100 Fraction)—HeLa IU₁-HF and/or -LF cells were cultured in MEM-HF and/or MEM-LF media for three days to about 80% confluence. After harvesting cells suspension using a rubber policeman, the suspension were centrifuged over silicone fluid to separate media and cells as shown before. Cells were incubated in 2 packed cell volumes of buffer A (10 mM Hepes, pH 7.9, containing 1.5mM MgCl₂, 10 mM KCl, for 10 min at 4°C, then they were lysed by 30 strokes of a Kontes all glass Douce homogenizer (B type pestle). After confirming cell lysis and recentrifugation (200 rpm for 10 min at 4°C), the supernatant was mixed with 0.11 volumes of buffer B (300 mM Hepes, pH 7.9, containing 30 mM MgCl₂, 1.4 M KCl), and centrifuged for 60 min at 100,000 x g (Beckman Type SW 55.Ti rotor). The protein concentration of supernatant was measured by BCA assay and 500 ml aliquots were frozen at -80°C (S-100 Fraction).

RNA-Protein Binding Assay—Binding reactions were carried out with 800 µg cytosonic extracts from HeLa IU₁-HF cells or -LF cells and 1 X 10⁶ cpm of ³²P-labeled 18-mer respectively in 10 mM Hepes, pH 7.6, containing 3 mM MgCl₂, 40 mM KCl, 5% glycerol, 600 ng/µl yeast tRNA, 1mM DTT in a final volume of 300 µl. After incubation at room temperature for 30 min, 500 units of RNase T1 and 20 µl of heparin solution (100 mg /ml) were added and incubation was continued for another 30 min.

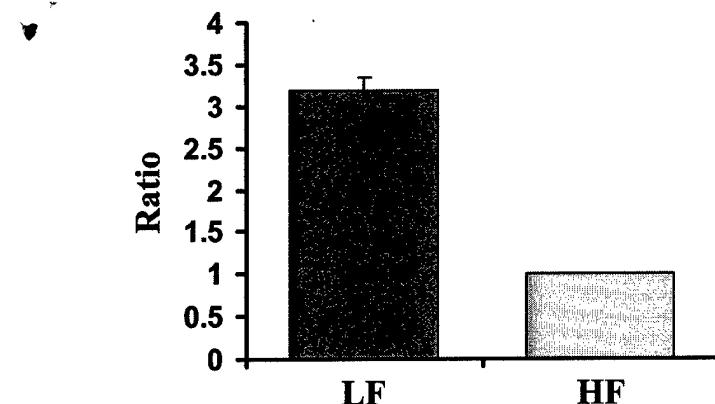
Gel-Shift Assay—Electrophoresis of 15 µl of RNA-Protein complexes was carried out using 6% native PAGE (60:1) and dried gel were autoradiographed at -80°C

UV Cross-linking of RNA-Protein Complexes—Rest RNA-Protein complexes were transferred to 24-well plate and irradiated from a distance of 1 cm by a UV lamp (300 nm, 70,000 µW/cm², Fotodyne Inc., New Berlin, WI) for 1 hour at 40C.

Specific Immunoprecipitation of UV cross-linked RNA-Protein Complexes—Twenty ml of nonimmune serum were incubated with UV cross-linked [³²P]RNA-Protein Complexes at 4°C for 2 h on a shaking platform. IgGsorb, 200 µl, was then added and incubated for 2 hours, followed by centrifugation at 13,600 x g for 5 minutes at 4°C to remove nonspecifically adsorbed proteins. The supernatant was aspirated and divided into 6 aliquots. After the addition of 20 µl of rabbit anti-hnRNPE1 antiserum or nonimmune serum (to 3 aliquots each), the final volume of each sample was brought up to 500 µl with D-PBS. Following incubation at 4°C for 18 hours, 200 µl of IgGsorb was added. After incubation for 2 hours and centrifugation at 13,600 x g for 5 minutes at 4°C, the pellets were washed with 1 ml of D-PBS for 3 times and resuspended in 0.48 ml D-PBS. An aliquot (100 µl) was mixed with 10 ml of counting cocktail and analyzed for radioactivity in a β-scintillation counter. The data obtained with non immune serum was subtracted from that obtained with anti-hnRNPE1 antiserum to derive values for specific [³²P]RNA-Protein Complexes. The remaining samples (from triplicates) were then combined, concentrated to 100 µl and [³⁵S]FR analyzed by SDS-PAGE and autoradiography.

Results

a) Counts Ratio Comparing RNA-Protein-Anti E1 from *LF* to One from *HF*



b) SDS-PAGE

